CRISPR/Cas9-Assisted Transformation-Efficient Reaction (CRATER) for near-perfect selective transformation

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Abstract

The CRISPR/Cas9 system has revolutionized genome editing by providing unprecedented DNA-targeting specificity. Here we demonstrate that this system can be also applied *in vitro* to fundamental cloning steps to facilitate efficient plasmid selection for transformation and selective gene insertion into plasmid vectors by cleaving unwanted plasmid byproducts with a single-guide RNA (sgRNA)-Cas9 nuclease complex. Using fluorescent and chromogenic proteins as reporters, we demonstrate that CRISPR/Cas9 cleavage excludes multiple plasmids as well as unwanted ligation byproducts resulting in an unprecedented increase in the transformation success rate from approximately 20% to nearly 100%. Thus, this CRISPR/Cas9-Assisted Transformation-Efficient Reaction (CRATER) protocol is a novel, inexpensive, and convenient application to conventional molecular cloning to achieve near-perfect selective transformation.

Introduction

Molecular cloning is a fundamental technique in molecular biology to produce plasmid constructs (Yoshida & Sato, 2009). Several methods currently exist to minimize or select against unwanted plasmid products created during ligation of inserts into vectors, including cross-incompatible sticky ends (Kim & Szybalski, 1988), X-gal blue/white screening (Ruther, 1980), dephosphorylation of backbone sticky ends (Bernard, 1996), the addition of antibiotics (Sutcliffe, 1978; Hershfield *et al.*, 1974), and agarose electrophoresis/gel extraction (Tabak & Flavell, 1978). However, in special circumstances existing methods may be insufficient to quickly, cheaply, and effectively screen for specific cloning products. This is especially true for plasmids with compatible sticky ends. Genes of interest may include restriction sites that would otherwise be used to create incompatible sticky ends. A plasmid vector also may simply not include multiple restriction sites with incompatible sticky ends. Unwanted byproducts are also difficult to control in situations where blunt ends are used (Sambrook & Russell, 2012).

The Cas9 protein is a component of the clustered, regularly interspaced, short palindromic repeats (CRISPR) system. The CRISPR/CRISPR-associated (Cas) system provides bacteria with acquired immunity by incorporating fragments of foreign DNA and using the transcribed CRISPR-RNA (crRNA) to guide the cleavage of matching dsDNA sequences (Garneau *et al*., 2010; Horvath & Barrangou, 2010; Bhaya *et al*., 2011; Wiedenheft *et al*., 2012). In type II CRISPR systems, a ternary complex of Cas9, crRNA, and trans-activating crRNA (tracrRNA) binds to and cleaves dsDNA sequences that match the crRNA and include a short protospacer-adjacent motif (PAM) recognized by Cas9 (Gasiunas *et al*., 2012; Qi *et al.*, 2013). In type II systems, the crRNA and tracrRNA can be combined into a single guide-RNA (sgRNA) that is sufficient to lead Cas9 to its target (Jinek e*t al*., 2012). Further, the PAM sequence

recognized by the *S. pyogenes* Cas9 is only three nucleotides in length (NGG), allowing this system to be easily adapted to recognize and cut a desired sequence (Mojica *et al.*, 2009).

The CRISPR/Cas system has previously been used to cleave the genomes of unwanted bacterial strains, showing high selectivity even among similar strains (Gomaa *et al.*, 2014). With the knowledge that Cas9 can also be used to cleave short (~24 bp) sequences during cloning experiments (Karvelis, Gasiunas, & Siksnys, 2013; Wang *et al.*, 2015), we investigated whether this system could be adapted to cleave unwanted ligation byproducts. We used the RFP BioBrick plasmid BBa_J04450 (Supplementary Table S1) as a starting vector and replaced the RFP insert with various genes of interest using restriction enzyme digestion and ligation, before transforming into *Escherichia coli*. We then quantified insertion efficiency based on the presence of fluorescent and chromogenic proteins in colonies and culture. We show, for the first time to our knowledge, that Cas9 and sgRNAs can be used to increase molecular cloning efficiency by cleavage of specific, undesired ligation byproducts; we call this novel technique CRISPR/Cas9-assisted transformation-efficient reaction (CRATER).

Results

CRATER enhances the success rate of selective transformation

We first sought to verify that *in vitro* Cas9 cleavage specifically selects for and purifies a desired plasmid product from an *in vitro* mixed pool. *E. coli* is known to have dramatically lower transformation efficiency for linear DNA compared with plasmids, due to intracellular exonuclease activity (Conley et al., 1986). To test the ability of CRATER to selectively prevent the transformation of multiple plasmid vectors in a mixed pool, we prepared a mixture of four different plasmids encoding color-producing proteins: RFP, eforRed, amilGFP, and meffBlue. We then designed four sgRNAs to selectively target each gene and added all combinations of

three sgRNAs to the mixtures of plasmids along with Cas9 nuclease (Fig. 1A). The efficiency of the Cas9-sgRNA complex against its target plasmid was verified by gel electrophoresis (Fig. 1B). We then chemically transformed the resulting reaction mix directly into DH5alpha *E. Coli* competent cells. As a result, the use of multiple sgRNAs in a single Cas9 reaction did not appear to interfere with successful target cleavage and therefore we observed highly monoclonal colonies after applying CRATER both upon visual inspection (Fig. 1C) and by quantification of percentage of desired colonies on transformed plates (Fig. 1D).

Cas9-sgRNA complex can selectively digest unwanted DNA in the ligation reaction

We next sought to demonstrate that CRATER can remove unwanted plasmids created during the ligation reaction. As an example, we began with a plasmid containing an RFP gene and double-digested the gene out of its vector backbone using EcoRI and SpeI. We then added a sticky end compatible preparation of the Ferulic Acid Decarboxylase (FDC) gene, a relatively longer gene that does not affect the growth of *E.coli* (Zago, Degrassi, & Bruschi, 1995), into the mix of EcoRI/SpeI digested RFP plasmid and performed ligation. Since we did not remove the RFP insert after digestion, we predicted that a significant amount of the vector backbone would ligate with the original RFP insert instead of the FDC gene due to the relatively short length. We then subjected the ligation reaction to CRATER with and without RFP-specific sgRNA, as a control. As a result, CRATER using RFP-specific sgRNA dramatically increased the percentage of colonies with the desired FDC insert when the transformed cells were plated and confirmed under visual inspection (Fig. 2A) as well as colony quantification. We found that CRATER can significantly remove unwanted re-ligated plasmid and increases the transformation success rate to near 100% ($p < 0.011$). To corroborate this result, we analyzed the same transformed cells grown overnight in LB liquid culture using flow cytometry. Applying CRATER reduced the

percentage of red colonies dramatically from 80% to 0.5% in flow cytometry (Fig. 2C) supporting our plate culture results (Fig. 2B).

Discussion

By experimenting with chromogenic and fluorescent protein gene inserts, we have demonstrated the ability of Cas9 to digest and prevent the undesired transformation of plasmids in mixed-ligation pools. This technique is one of the many new applications of the recently discovered CRISPR/Cas system and can be used to augment existing methods for manipulating recombinant DNA. CRATER will save time when manipulating DNA and constructing plasmids. Particularly in cases when transformation efficiency is very low, such as when transforming large genes (>10 kb) into plasmids (Hanahan, 1983; Inoue, Nojima, & Okayama, 1990), increasing efficiency will reduce the number of colony PCR amplifications and sequencing required to find a colony with the desired insert. In addition to streamlining low-efficiency cloning experiments, CRATER could allow for tighter control of gene order and orientation during multi-component ligations. Designing sgRNAs that target reverse-oriented inserts could facilitate gene orientation control even when restriction enzymes give rise to complementary sticky ends. Moreover, sgRNA specificity could allow for this same level of control when inserting multiple genes into a single vector. By designing sgRNAs that target unwanted orders and orientations of gene inserts, efficient construction of complex plasmids is made possible.

As Gibson assembly (Gibson *et al.*, 2009) can also be used to efficiently construct plasmids with multiple genes, we wanted to discern whether the CRATER method was comparable to Gibson in terms of cost. To this end, we conducted a market study of biotechnology firms including New England Biolabs, Life Technologies, ElimBio, DNA2.0, and

Integrated DNA Technologies (IDT). As of August 2015, the market price of a Gibson Assembly Kit was $630.00, with primers pairs ranging from $10 to $30 per pair. The combined market price of sgRNA synthesis materials and Cas9 digestion materials ranged from $708.80 to $874.30, assuming only one type of sgRNA was needed. Each additional sgRNA would cost $47.40. Both Gibson assembly and Cas9 digestion costs are for 50 reactions worth of materials, while the sgRNA synthesis cost is for 15-75 μg of sgRNA. Based on these estimates, CRATER appears to be roughly equivalent in cost to Gibson assembly, and is particularly useful for when fragment termini instability or repetitive DNA sequences prevent Gibson from being used, Gibson primer design results in primer dimer or hairpin formation (Hillson, 2011), or when only one stock sgRNA is needed to prevent a particularly common unwanted ligation product. A good example of this last case is plasmid backbones with standardized restriction sites, such as the PSB1C3 backbone used in the iGEM BioBrick Registry. This plasmid contains XbaI and SpeI restriction sites, so sgRNAs that target XbaI/SpeI-scarred re-ligated plasmids could be mass-produced and distributed with the standard backbone.

Methods

Plasmids. All plasmids were obtained from the BioBrick Registry. BioBrick numbers, sizes, and descriptions are provided in Supplementary Table 1.

DNA Quantification and Sequencing. DNA concentrations were determined using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Sequencing of DNA samples was completed by Elim Biopharmaceuticals, Inc. (Hayward, CA, USA).

sgRNA Preparation. The tracrRNA reverse template primer along with crRNA forward primers were ordered from Elim Biopharmaceuticals, Inc. The 10 PCR primers used are shown in Supplementary Table 2. Single guide-RNA templates were PCR amplified from these primers in a 50 µL reaction, with initial denaturation at 98ºC for 30 seconds, annealing at 62ºC for 15 seconds, and elongation at 72ºC for 10 seconds, repeating for 10 cycles. The templates were isolated via the Epoch Life Sciences Inc. (Missouri City, TX, USA) PCR cleanup protocol. Transcription of sgRNAs was accomplished using the HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA). Last, sgRNAs were purified using the Life Technologies (Carlsbad, CA, USA) RNA extraction protocol.

CRISPR/Cas9-Assisted Transformation-Efficient Reaction (CRATER). Single restriction enzyme digestion with PstI (NEB catalog: #R3140) was accomplished using the New England Biolabs protocol. The MinElute Reaction Cleanup Kit (Qiagen, Limburg, Netherlands) was used to purify restriction enzyme digests. Restriction enzyme digests were ligated using the New England Biolabs T4 Ligation protocol. The RFP and chromogenic plasmids were digested using the New England Biolabs *in vitro* Cas9 Digestion protocol, with the modification that 2 µL of 1µM Cas9 nuclease was added to the reaction instead of 1µL. When multiple sgRNAs were added to the same mixture, a 300 nM solution containing the sgRNAs was prepared in advance, and 3 µL of this solution was added to the reaction mixture.

Transformation. The PSB1C3 BioBrick plasmid backbone was used as a vector with chloramphenicol selection, and insert RFP and GFP genes were taken from the Biobrick Registry (BBa_J04450 and BBa_I13522, respectively). *E. coli* NEB5α chemically competent cells were purchased from New England Biolabs. Transformants were plated on LB plates with

chloramphenicol selection by adding 50 µL of transformant mixture to the plate and spreading evenly using glass beads. 20 µL of transformants were also incubated in 3 mL of LB broth with chloramphenicol selection to analyze on the flow cytometer. Both plates and liquid cultures were grown at 37ºC for ~16 hours.

Plate Imaging. Plates were photographed using a Canon EOS 5D Mark II, Canon 100mm f/2.8 macro lens, and a fluorescent white light box.

Fluorescent Measurement. Liquid cultures of transformed *E. coli* were analyzed using Life Technologies Attune NxT Acoustic Flow Cytometer. 150 µL of each 5X dilute liquid culture was drawn at 12.5 µL/second until at least 500,000 events of single cells were collected (Supplementary Figure S2).

References

Bernard, P. Positive selection of recombinant DNA by CcdB. *Biotechniques.* **21,** 320–323 (1996).

Bhaya, D., Davison, M., & Barrangou, R. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* **45,** 273-97 (2011).

Conley, E. C., Saunders, V. A., & Saunders, J. R. Deletion and rearrangement of plasmid DNA during transformation of *Escherichia coli* with linear plasmid molecules. *Nucleic Acids Res.* **14,** 8905-8917 (1986).

Garneau, J. E. *et al.* The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature.* **468,** 67–71 (2010).

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. USA.* **109,** 2579-2586 (2012).

Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods.* **6,** 343–345 (2009).

Gomaa, A.A. *et al*. Programmable removal of bacterial strains by use of genome-targeting CRISPR-Cas systems. *MBio.* **5,** e00928–13 (2014).

Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557–580 (1983).

Hershfield, V., Boyer, H. W., Yanofsky, C., Lovett, M. A., & Helinski, D. R. Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA. *Proc. Natl. Acad. Sci. USA.* **71,** 3455-3459 (1974).

Hillson, N. J. DNA assembly method standardization for synthetic biomolecular circuits and systems. In *Design and Analysis of Biomolecular Circuits: Engineering Approaches to Systems and Synthetic Biology*, (eds. Koeppl, H., Setti, G., di Bernardo, M., & Densmore, D., 2011).

Horvath, P. & Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. *Science.* **327,** 167–170 (2010).

Inoue, H., Nojima, H., & Okayama, H. High efficiency transformation of *Escherichia coli* with plasmids. *Gene.* **96,** 23–28 (1990).

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. **337,** 816-821 (2012).

Karvelis, T., Gasiunas, G. & Siksnys, V. Programmable DNA cleavage *in vitro* by Cas9. *Biochem. Soc. Trans.* **41,** 1401–1406 (2013).

Kim, S. C. & Szybalski, W. Amplification of cloned DNA as tandem multimers using *Bsp*MI-generated asymmetric cohesive ends. *Gene*. **71,** 1-8 (1988).

Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J., & Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*. **155,** 733-740 (2009).

Qi, L.S. *et al.* Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell.* **152,** 1173–1183 (2013).

Ruther, U. Construction and properties of a new cloning vehicle, allowing direct screening for recombinant plasmids. *Mol. Gen. Genet.* **178,** 475-477 (1980).

Sambrook, J. & Russell, D. W. *Molecular Cloning: A Laboratory Manual, 4th edn* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 2012).

Sutcliffe, J. G. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. *Proc. Natl. Acad. Sci. USA.* **75,** 3737-3741 (1978).

Tabak, H. F. & Flavell, R. A. A method for the recovery of DNA from agarose gels. *Nucleic Acids Res.* **5,** 2321-2332 (1978).

Wang *et al.* CRISPR/Cas9 nuclease cleavage combined with Gibson assembly for seamless cloning. *BioTechniques* **58,** 161-170 (2015).

Wiedenheft, B., Sternberg, S. H., & Doudna, J. A. RNA-guided genetic silencing systems in bacteria and archaea. *Nature*. **482,** 331-338 (2012).

Yoshida, N. & Sato, M. Plasmid uptake by bacteria: a comparison of the methods and efficiencies. *Appl. Microbiol. Biotechnol.* **83,** 791-798 (2009).

Zago, A., Degrassi, G., & Bruschi, C. V. Cloning, sequencing, and expression in *Escherichia coli* of the *Bacillus pumilus* gene for ferulic acid decarboxylase. *Appl. Environ. Microbiol.* **61,** 4484-4486 (1995).
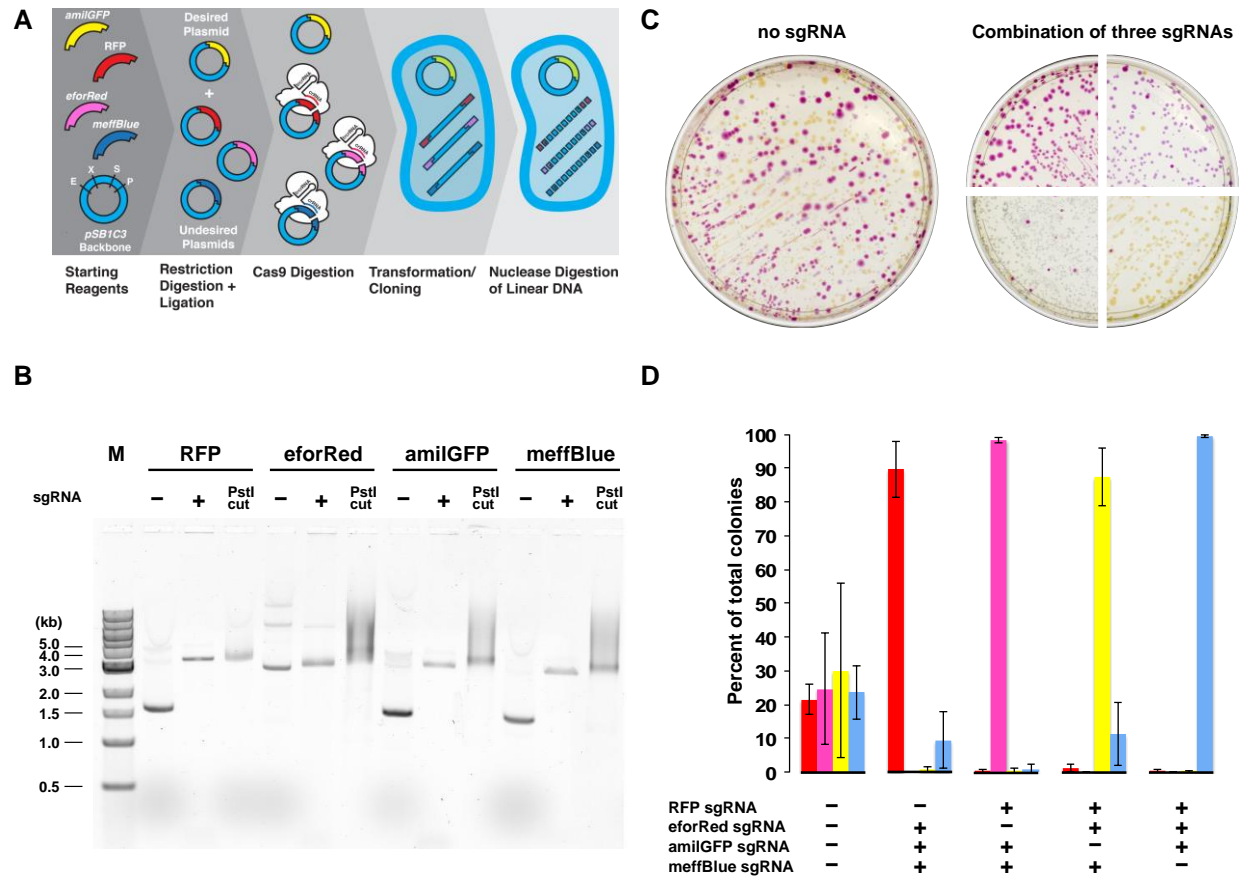
Figure 1. *in vitro* **CRISPR/Cas9 nuclease cleavage enhances transformation selectively.** (A) Overview of CRATER method selecting for a chromogenic plasmid in a mixed pool. After ligation of chromogenic genes into the pSB1C3 plasmid backbone, plasmids are combined with recombinant *S pyogenes* Cas9 nuclease and mixture of sgRNAs targeting the unwanted gene inserts. The targeted plasmids are cleaved *in vitro* into linear form, but leaving the desired plasmid intact. Digested products can be directly transformed into *E.coli* competent cells. Intracellular exonucleases further cleave the linear DNA, leaving only the desired transformants (in this case *E.coli* with *amilGFP* plasmid). (B) Gel electrophoresis of plasmids with and without (-) sgRNA demonstrating cleavage of plasmids to linear DNA. The expected length of linearized DNA for each plasmid is ~2.8 kb. (C) *E. coli* transformants expressing chromogenic proteins after transformation with a mixture of four different plasmids with sgRNA-free control (left) and

with CRATER using a conbination of three sgRNAs. (D) Bar graph represent the plasmid selectivity based on the percent of colonies for each chromogenic protein. Absent bars represent no colonies. Error bars represent standard deviation with n = 3. CFUs are shown in Supplemental Table S3.

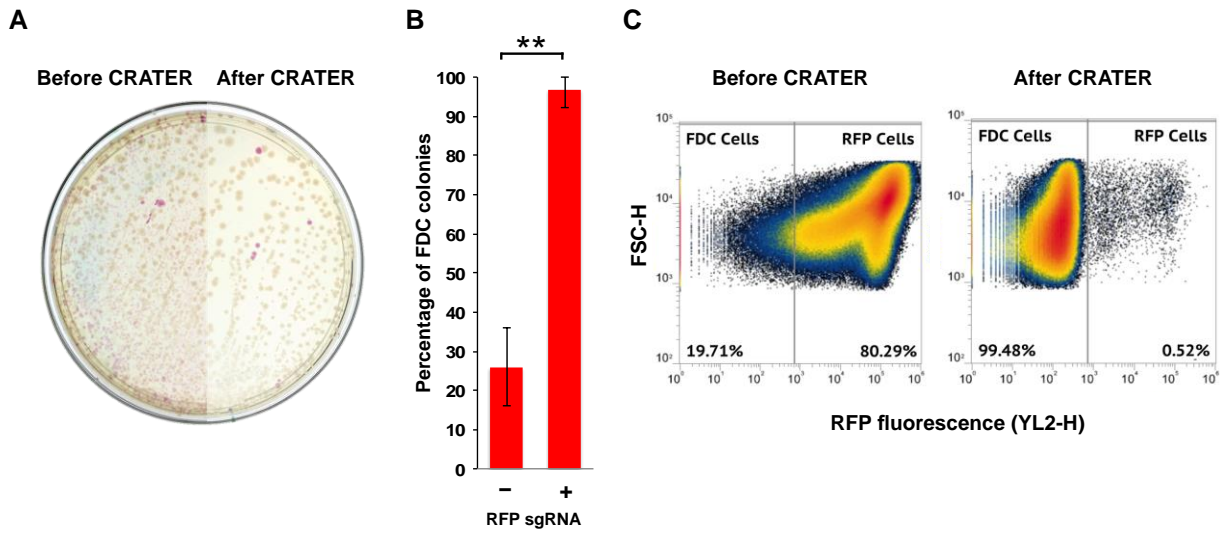**Figure 2. CRATER can selectively prevent the transformation of unwanted re-ligation product.** (A) Outcome of CRATER with (right) and without (left) RFP gene targeting sgRNA. Competent *E. coli* was transformed with FDC insert (1.6 kbp) competing against RFP insert (678 bp) leftover from RFP plasmid digestion products. White colonies represent successful ligation of the FDC gene, while red colonies represent the unwanted RFP re-ligation product. (B) Percentage of total colonies on the plates with each insert, n = 2. Error bars represent the standard deviation, p-value < 0.011 according to one-way ANOVA test. (C) Flow cytometry data of the transformants shown in Fig. 2A after growing overnight in liquid LB culture under antibiotic selective pressure. At least 500,000 individual *E.coli* cells are plotted on the 2D plot with forward scatter height signal (FSC-H) on the x-axis and RFP fluorescence signal (YL2-H) on the y-axis in log scales.

Author Contributions

L.J.R. conceived the experiments. L.J.R., K.F., A.J.M., and J.D.S. oversaw the experiments and edited the manuscript. D.T.G., K.A.T., J.R.T., T.D.D., T.J., D.K., F.T., and D.X. performed the experiments and analyzed the data. D.T.G., D.K., K.A.T., J.R.T., C.C., E.L., and T.N. wrote the manuscript. All authors approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

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